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(54) Title: LEUKOCYTE ADHESION MOLECULE-1 (LAM-1) AND LIGAND THEREOF

(57) Abstract

A leukocyte-associated cell surface protein LAM-1 (leukocyte adhesion molecule-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; the specific domains of the LAM-1 protein; ligand binding fragments of the LAM-1 protein; and the genomic DNA sequences encoding the LAM-1 protein and the specific domains of LAM-1 are disclosed. Also disclosed are methods and agents for detecting, identifying and characterizing the LAM-1 ligand. The LAM-1 protein or a ligand binding fragment thereof or an antagonist to the LAM-1 protein or ligand binding fragment thereof are used in methods of detecting sites of inflammation or disease in a human patient. They are also used in therapeutic compositions in methods of treating a patient suffering from a leukocyte-mobilizing condition.

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# LEUKOCYTE ADHESION MOLECULE-1 (LAM-1) AND LIGAND THEREOF

This application is a continuation-in-part of Tedder, U.S. Patent Application Serial No. 07/700,773, filed May 15, 1991, the whole of which is hereby incorporated by reference herein.

Part of the work leading to this invention was made with United States Government funds. Therefore, the U.S. Government has certain rights in this invention.

## FIELD OF THE INVENTION

This invention relates to human leukocyte-associated cell surface proteins and particularly to leukocyte adhesion molecule-1 (LAM-1).

## BACKGROUND OF THE INVENTION

Genes exclusively expressed by one cell lineage, but not by others, often define the function of that cell population. The generation of genes by the assembly of functionally independent domains has occurred frequently as new genes have evolved to encode proteins with new functions. An inducible endothelial-leukocyte adhesion molecule (ELAM-1), having several functionally independent domains, is expressed on the surface of cytokine-treated endothelial cells. This molecule is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining (Bevilacqua et al., Proc. Natl. Acad. Sci. USA 84:9238 (1987)). A granule membrane protein found in platelets and endothelial cells, termed GMP-140, has been cloned and is homologous with ELAM-1 (Johnston et al., Blood Suppl. 1 72:327A (1988)).

## SUMMARY OF THE INVENTION

The invention generally features a leukocyte-associated cell surface protein LAM-1 (leukocyte adhesion molecule-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; the specific domains of the LAM-1 protein; the genomic DNA sequences encoding the LAM-1 protein and the specific domains of LAM-1; methods of detecting the presence of a LAM-1 ligand; ligand binding fragments of LAM-1; methods of developing an antagonist to LAM-1 or LAM-1 ligand function; and methods of treating a human patient suffering from a leukocyte-mobilizing condition.

In one aspect the invention features a method of detecting a site of inflammation or a diseased state in a human patient that includes administering to the patient a pharmaceutical composition comprising a detectable amount of a labeled LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance, and detecting the label on the LAM-1 protein.

In preferred embodiments the LAM-1 protein, or ligand binding fragment, is labeled with a radionuclide, a paramagnetic isotope, or a radiopaque label. The LAM-1 protein, or ligand binding fragment, can also be joined to a carrier protein, preferably an immunoglobulin heavy chain constant region or non-ligand binding portions of a selectin molecule.

In another aspect the invention features an imaging agent for imaging a site of inflammation or a diseased state in a human patient that includes LAM-1 protein or a ligand binding fragment thereof labeled with a detectable label and suspended in a pharmaceutically acceptable carrier substance. In preferred embodiments the detectable label comprises a radionuclide, a paramagnetic isotope, or a radiopaque label. The LAM-1 protein, or ligand binding fragment, in the imaging agent can also be joined to a carrier protein, preferably an immunoglobulin heavy chain constant region or non-ligand binding portions of a selectin molecule.

In another aspect the invention features a method of treating a human patient suffering from a leukocyte-mobilizing condition that includes administering to the patient a therapeutic composition comprising a therapeutic amount of LAM-1 protein or ligand binding fragment, or antagonist to the LAM-1 protein or specific ligand binding fragment, in a pharmaceutically acceptable carrier substance. Preferably, the patient is suffering from cancer, tissue damage or an autoimmune disorder. Alternatively, the patient is an organ or tissue transplant recipient. In preferred embodiments the LAM-1 protein, or ligand binding fragment, is joined to a therapeutic agent, which is preferably either a chemotherapeutic drug or an anti-inflammatory agent. Alternatively, the method further includes administering to the patient a therapeutic amount of a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or a soluble fragment of the cell surface molecule. (The cell surface molecules or soluble fragments are those capable of participating in the adhesion of leukocytes to endothelial surfaces.)

As used herein the term "antagonist to LAM-1" includes any agent which interacts with LAM-1 and interferes with its function, e.g., antibody reactive with LAM-1 or any ligand which binds to LAM-1. The term "identify" is intended to include other activities that require identification of an entity, such as isolation or purification. The term "essentially purified" refers to a protein or nucleic acid sequence that has been separated or isolated from the environment in which it was prepared or in which it naturally occurs.

Leukocyte-associated cell surface protein LAM-1 plays an important role in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. The LAM-1 protein or ligand binding fragments or specific domains thereof, or oth r molecules that interfere with leukocyte adhesion and function, can be used

therapeutically to inhibit the inflammatory response and to treat such conditions as tissue damage and metastasis of cancer cells. The assays for LAM-1 function are highly reproducible, easily performed, and allow the precis quantitation of the extent of the LAM-1 interaction with ligand or ligands. Thus, they permit quantitation of the extent of involvement of different receptor systems in adhesion and the easy identification of antagonists to LAM-1 function.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the drawings, Figs. 1A and 1B show the structure of LAM-1 cDNA clone;

Fig. 2 shows the cDNA nucleotide sequence and also shows the amino acid sequence of LAM-1;

Figs. 3A, 3B, and 3C show the homologies of LAM-1 with other proteins;

Figs. 4A, 4B, and 4C show the restriction map and the exon-intron organization of the *lyam-1* gene; and

Fig. 5 shows the nucleotide sequence of exons II through X of the *lyam-1* gene.

The leukocyte adhesion molecule-1 (LAM-1), also called lymphocyte-associated molecule-1, is expressed by human lymphocytes, neutrophils, monocytes and their precursors and is a member of the selectin family of cellular adhesion/homing receptors which play important roles in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. LAM-1 combines unrelated domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins.

A ligand for LAM-1, induced on cultur d human endothelium following activation with specific inflammatory

cytokines, is involved in the initial attachment of homing leukocytes to the endothelial cells.

Identification and characterization of the LAM-1 protein.

cDNA encoding the LAM-1 protein was initially identified as follows. B cell-specific cDNAs were isolated from a human tonsil cDNA library (ATCC #37546) using differential hybridization with labeled cDNAs derived from either B cell (RAJI) RNA or T cell (HSB-2) RNA (Tedder et al., Proc. Natl. Acad. Sci. USA 85:208-212 (1988)). Positive plaques were isolated and cloned, and the cDNA inserts were subcloned into the plasmid pSP65 (Promega, Madison, WI). Nucleotide sequences were determined using the method of Maxam and Gilbert (Meth. Enzymol. 65:499 (1980)). Gap penalties of -1 were assessed during homology analysis for each nucleotide or amino acid in the sequence where a gap or deletion occurred. One of the 261 RAJI+ HSB2- cDNA clones isolated, B125, contained a 1.90 kilo base pair (kb) cDNA insert that hybridized with a 2.4 kb RNA species found in several B cell lines (Tedder et al., supra). However, B125 did not hybridize with any of the other RAJI+ RSB2- clones or with mRNA from several T cell lines. The B125 cDNA clone was characterized by restriction mapping and nucleotide sequence determination. A near-full-length 2.3 kb cDNA that hybridized with B125 was isolated, sequenced, and termed pLAM-1.

The expression of LAM-1 mRNA by cell lines of lymphoid and non-lymphoid origin was examined. Northern blot analysis revealed that LAM-1 cDNA hybridized strongly to a 2.6 kb RNA species and weakly to a 1.7 kb RNA species in poly(A)+ RNA isolated from the B cell lines RAJI, SB, Laz-509, and GK-5. However, RNA isolated from two pre-B cell lines (Nalm-6, PB-697), three B cell lines (Namalwa, Daudi, BJAB), five T cell lines (CEM, Hut-78, HSB-2, Molt-15, Molt-3), a myelomonocytic cell line (U937 and U937 cultured with LPS) and erythroleukemic K-562 cell line did not hybridize with LAM-1

cDNA suggesting that expression of this gene was preferentially associated with B lymphocytes. Neutrophils expressed LAM-1 mRNA but had a relatively lower amount of transcript among total mRNA when compared with the RAJI cell line or blood T lymphocytes. LAM-1 cDNA has also been used to transfer expression of LAM-1 to cells that do not express the gene.

As shown in Fig. 1A, a restriction map was constructed by the standard single, double or triple digestions of pLAM-1. The coding region is shown in black. Arrows indicate the direction and extent of nucleotide sequence determination and the open circles indicate 5′-end labeling. In Fig. 1B, a schematic model of the structure of the LAM-1 mRNA is shown, thin lines indicate 5′ and 3′ untranslated sequences (UT), while the thick bar indicates the translated region. The boxes represent the lectin-like and epidermal growth factor (EGF)-like domains and the two short consensus repeat (SCR) units. The open box indicates the transmembrane (TM) region.

pLAM-1 contains an open reading frame that could encode a protein of 372 amino acids as shown in Fig. 2. The numbers shown above the amino acid sequence designate amino acid residue positions. The numbers to the right indicate nucleotide residue positions. Amino acids are designated by the single-letter code, and * indicates the termination codon. The boxed sequences identify possible N-linked glycosylation sites. Hydrophobic regions that may identify signal and transmembrane peptides are underlined. The amino acid sequence of LAM-1 indicates a structure typical of a membrane glycoprotein. The mature LAM-1 protein has an extracellular region of about 294 amino acids containing 7 potential N-linked carbohydrate attachment sites. LAM-1 has a cytoplasmic tail of 17 amino acids containing 8 basic and 1 acidic residues. The processed LAM-1 protein has a Mr of at least 50,000 and can be isolated by conventional techniques, such as affinity column chromatography with antibody or ligand, from cell lines that normally express

this receptor or from transfected cell lines. Or the protein can be synthesized by in vitro translation of the LAM-1 cDNA.

LAM-1 combines domains homologous to domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins. The extracellular region of LAM-1 contains a high number of Cys residues (7%) with a general structure as diagrammed in Fig. 1B. As indicated in Fig. 3, segments of homologous proteins are shown with the amino acid residue numbers at each end. Homologous amino acids are shown in boxes. Gaps (-) have been inserted in the sequences to maximize homologies. The first 157 amino acids of the protein (Fig. 3A) were homologous with the low-affinity receptor for IgE (Kikutani et al., Cell 47:657 (1986)), the asialoglycoprotein receptor (Spiess et al., Proc. Natl. Acad. Sci. USA 82:6465 (1985)) and several other carbohydrate-binding proteins (Drickamer et al., J. Biol. Chem. 256:5827 (1981); Ezekowitz et al., J. Exp. Med. 167:1034 (1988); Krusius et al., J. Biol. Chem. 262:13120-13125 (1987); and Takahashi et al., J. Biol. Chem. 260:12228 (1985)). The amino acids conserved among all animal-lectin carbohydrate recognition domains are indicated (*). Although the sequence homologies were less than 30%, all the invariant residues found in animal-lectin carbohydrate-recognition domains were conserved (Drickamer, J. Biol. Chem. 263:9557 (1988)). The lectin domain included amino acid residues 42-170 given in Fig. 2.

The next domain of 36 amino acids, at residues 171-206 shown in Fig. 2, was homologous (36-39%) with epidermal growth factor (EGF) (Gregory, Nature 257:325 (1975)) and the EGF-like repeat units found in Factor IX (Yoshitake et al., Biochem. 25:3736 (1985)) and fibroblast proteoglycan core protein (Krusius et al., supra) (Fig. 3B).

Immediately following these domains were two tandem domains of 62 amino acids each, given by residues 207-269 and 270-331 of Fig. 2, that were homologous with the short consensus repeat unit (SCR) that comprises the IL-2 receptor

(Leonard et al., Nature 311:626 (1984)), Factor XIII (Ichinose et al., Biochem. 25:4633 (1986)) and many C3/C4 binding proteins (Klickstein et al., J. Exp. Med. 165:1095 (1987); and Morley et al., EMBO J. 3:153 (1984)). In contrast with all of the previously described SCR that contain four conserved Cys residues, these two SCR possessed six Cys residues. The four conserved Cys residues found in all SCR are indicated in Fig. 3C by (*); the additional conserved Cys found in LAM-1 are indicated by (+). Of the multiple SCR present in each of these proteins, the SCR with the highest homology to LAM-1 is diagrammed (Fig. 3C). A 15 amino acid spacer followed the short consensus repeat units, preceding the transmembrane domain.

The structure of the *lyam-1* gene, which encodes the LAM-1 protein, was determined by isolating overlapping genomic DNA clones that hybridized with a LAM-1 cDNA probe. The *lyam-1* gene spans greater than 30 kb of DNA and is composed of at least 10 exons. The 5′ end of the LAM-1 mRNA was mapped by primer extension analysis, revealing a singl initiation region for transcription. Exons II through X contain translated sequences; exon II encodes the translation initiation codon, residue 14 shown in Fig. 2; exon III encodes the leader peptide domain, residues 15-41; exon IV encodes the lectin-like domain, residues 42-170; exon V encodes the epidermal growth factor-like domain, residues 171-206; exons VI and VII encode the short consensus repeat unit domains, residues 207-269 and 270-331; exon VIII encodes the transmembrane region, residues 332-373; exon IX encodes seven amino acids containing a potential phosphorylation site, residues 374-380; and exon X encodes the five remaining amino acids of the cytoplasmic tail and the long 3′ untranslated region.

The pLAM-1 cDNA was labeled with $^{32}P$ and used as a probe to isolate hybridizing DNAs from a human leukocyte genomic DNA library. Approximately 1 X $10^6$ plaques were screened, and 13 plaques that hybridized with the cDNA probe were

identified and isolated. Seven of these clones were found to contain inserts with unique restriction enzyme maps representing overlapping genomic fragments spanning at least 30 kb. These inserts, LAMG-17, -19, -20, -28, -35, -37, and -47, were further digested and subcloned into plasmids. Detailed restriction maps of these subclones were made and compared to those of intact inserts to determine their correct locations (Figs. 4A and 4B).

The correctness of the restriction map was verified with Southern blot analysis. DNA isolated from two B cell lines, BL and BJAB, and one T cell line, HSB-2, was digested to completion with *Bam* HI, *Bal* II, or *Pvu* II, size-fractionated, and transferred onto nitrocellulose. This filter was probed with the LAM-1 cDNA clone, pLAM-1. All genomic fragments derived from endonuclease digested DNA hybridized with cDNA probe to generate hybridizing bands of the appropriate siz .

The pLAM-1 cDNA clone encodes an 85-bp 5′ untranslated region. An oligonucleotide homologous with the 5′ sequence of the pLAM-1 cDNA was used as a probe for primer extension analysis. This oligonucleotide was hybridized with poly ($A^+$) RNA isolated from the human B cell line RAJI, the LAM-1 negative human B cell line Namalwa, the mouse pre-B cell line A20, and yeast tRNA as a control. Complementary DNA was synthesized by extending the primer with reverse transcriptase. The major primer extension product obtained using the human LAM-1 positive B cell line RNA was extended 126 nucleotides beyond the translation initiation site. There was a single cluster of transcription initiation sites for the *lyam-1* gene apparent in the reaction with RAJI RNA that was not found with the LAM-1 negative Namalwa RNA. Several primer extension products of size similar to those of the human B cell line RNA were obtained with mouse B cell RNA. Therefore, murine B cells may express an RNA species that cross-hybridizes with the oligonucleotide probe used. No primer extension products were obtained in the yeast tRNA control reactions.

The relationship of the primer extension results to the cloned LAM-1 cDNAs and the most 5′ exon of *lyam-1* isolated was used to determine the nucleotide sequence of the exon that encodes the translation initiation AUG codon. This exon ends immediately after the site that encodes the translation initiation codon (Fig. 5) and overlaps precisely with the pLAM-1 cDNA sequence. The length of the cDNA clone obtained by Bowen et al., J. Cell Biol. 109:421-427 (1989) agrees precisely with the primer extension results except for two nucleotides. However, 15 nucleotides before the 5′ end of the cDNA the sequence diverges from the genomic sequence at a site homologous with the 3′ splice acceptor site consensus sequence. Therefore, it is most likely that this 15-bp region is derived from the exon upstream of this potential splice site. Thus, the primer extension results indicate that exon I would most likely be composed of 15 or fewer base pairs.

A 15-bp oligonucleotide homologous with the 5′ nucleotides present in the cDNA clone of Bowen et al., supra but not encoded by exon II, was used to probe the 10 kb of cloned DNAs 5′ of exon II; however, specific hybridization was not detected by Southern blot analysis. Under the conditions necessary for hybridization of this oligonucleotide, significant cross-hybridization occurred with λ-DNA, making it difficult to use this oligonucleotide to isolate the first exon from a λ-based genomic library.

These results suggest that the exon which encodes the translation initiation site is the second exon of the *lyam-1* gene (Fig. 4C). Consistent with this, the 900 bp upstream of exon II did not contain any apparent "TATA" or "CCAAT" sequences frequently found in promoter regions of eukaryotic genes (Fig. 5). Therefore, it is likely that the transcription initiation region and exon I are further than 10 kb upstream from exon II of the *lyam-1* gene. S1 nuclease protecti n analysis was carried out using the 5′ region of exon II as a labeled probe for hybridization with poly ($A^+$)

RNA from RAJI, Namalwa, and A20 cells. Two mRNA species were protected in the RAJI mRNA, while no S1 protection was provided by the other RNAs. The length of these fragments was consistent with differential splicing at the two potential CAG/N splice sites located within the potential splice acceptor site in exon II. It is therefore likely that the transcription initiation region has not been identified.

The majority of the exons were localized by comparison of the restriction enzyme maps of the genomic clones and the pLAM-1 cDNA. In cases where this method did not provide definitive results, subcloned DNA fragments were digested with selected restriction enzymes, electrophoresed through agarose gels, and transferred to nitrocellulose. Fragments containing exons were identified by Southern blot analysis using labeled cDNA or oligonucleotide probes. The exon that encodes the 3′ untranslated region of the LAM-1 cDNA was not contained within the 30 kb of isolated DNA fragments. Therefore, a labeled 0.9-kb *Dra* I fragment containing most of the 3′ untranslated region of the pLAM-1 cDNA was used as a probe to identify a homologous 3.2-kb fragment generated by complete *Eco* RI digestion of genomic DNA. *Eco* RI-digested genomic DNA fragments of this size were used to make a partial λ-gt11 genomic library from which the 3.2-kb *Eco* RI fragment was cloned. This 3.2-kb fragment did not overlap with the previously isolated genomic DNAs.

The exact boundaries of the exons were determined by nucleotide sequence analysis. From this analysis, nine exons were identified which make up the entire pLAM-1 cDNA. Exon II encodes the translation initiation codon, and exon III encodes the leader domain of the LAM-1 protein (Fig. 5). Each of the lectin-like, epidermal growth factor-like, transmembrane, and short consensus repeat domains was encoded by a separate exon. The smallest exon, IX, is 19 bp in length and may encode a carboxyl-terminal phosphorylation cassette. Th last 5 amino acids of the LAM-1 protein and the 3′ untranslated region which includes the poly(A)

attachment site, AATAAA, are encoded by exon X as shown in Fig. 5. The nine exons which encode pLAM-1 were split inside codons in all cases, except the junction between exons II and III. In each instance, the consensus sequences of 5' donor splice sites and 3' acceptor splice sites were adhered to. Nucleotide sequence polymorphisms within the coding region were observed between the genomic clones containing exon V that encoded SCR I and the pLAM-1 clone at cDNA nucleotide positions 741 and 747 (A to G), leading to a coding change from Asn to Ser in both cases, and at position 816 (A to G) changing the Glu to a Gly.

Detection, identification and characterization of the LAM-1 ligand.

**MATERIALS AND METHODS**

*Endothelial cell cultures.* Human umbilical vein endothelial cells (HUVEC) were isolated from two to five umbilical cord veins, pooled and established as primary cultures in medium 199 (M199, Gibco BRL, Grand Island, NY) containing 20% FCS (Gibco BRL). HUVEC were serially passaged and maintained using M199 supplemented with 10% FCS, endothelial cell growth factor (50μ/ml, Biomedical Technologies, Inc., Stoughton, MA) and porcine intestinal heparin (50 μg/ml; Sigma, St. Louis, MO) in tissue culture flasks precoated with 0.1% gelatin. For experimental use, passage 2 to 3 of HUVEC were plated on microscopic slides coated with 0.1% gelatin and growth within a 2.2 cm diameter circle delineated by a ring of 12 M polysiloxane.

*Leukocyte isolation.* Blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal volunteers. Neutrophils were isolated by centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA) followed by lysis of red blood cells with ice-cold hypotonic 0.2% (w/v) NaCl solution. Cells were kept on ice until use.

*Mab and PPME.* Murine monoclonal antibodi s (mAb) used were those directed against distinct epitopes of LAM-1, anti-LAM1-1, -3, -4, -5, -6, -7, -8, -10 and -11, all of the $IgG_1$ isotype, and were used as diluted ascites fluid (1:100) (Spertini et al., Leukemia 5:300 (1991); Spertini et al., J. Immunol. (in press) (1991)). The anti-ELAM-1 mAb H18/7 ($IgG2_a$), which inhibits the adhesion of neutrophils to activated endothelium, was used as purified $F(ab')_2$ fragments (25 μg/ml) (Bevilacqua et al., Proc. Natl. Acad. Sci. USA 84:9238 (1987); and Bevilacqua et al., Science 243:1160 (1989)); the anti-CD29 (VLA-ß chain, 4B4, $IgG_1$) (Nojima et al., J. Exp. Med. 172:1185 (1990)) and anti-CD18 (H52, $IgG_1$) (Hildreth et al., J. Immunol. 134:3272 (1985)) mAb were used as diluted ascites fluid (1:100). Anti-HLA class I mAb (W6/32, $IgG2_a$) was used as purified $F(ab')_2$ fragments (25 μg/ml). The anti-VCAM-1 mAb HAE-2 (Human Activated Endothelium-2; $IgG_1$) was generated by the fusion of Ag8.653 myeloma cells with spleen cells from BALB/c mice that were repeatedly immunized with HUVEC stimulated for 6 hours with 100 U/ml TNF-α. The specificity of the HAE-2 mAb was determined by its ability to bind recombinant VCAM-1 in a radioimmunoassay (W. Newman, Otsuka America Pharmaceuticals, Inc., Rockville, MD) (Polte et al., Nucl. Acids Res. 18:5901 (1990)) and its immunoprecipitation of a surface iodinated molecule of 110 $M_r$ from activated HUVEC. HAE-2 was used as diluted ascites fluid (1:100). Phosphomannan monoester core complex (PPME) from the yeast *Hansenula holstii* cell wall (provided by Dr. M. Slodki, USDA, Peoria, IL) was used at a final concentration of 3 μg/ml. Heparin (Sigma) was used as a control anionic polysaccaride at a final concentration of 3 μg/ml.

*Activation of HUVEC.* Confluent endothelial monolayers were stimulated for 2 to 24 hours with recombinant human (rh) TNF-α (100 U/ml; a gift from Asahi, Inc., Japan), rh IL-1ß (10 U/ml; a gift from Biogen, Inc., Cambridge, MA), LPS (1 μg/ml; *Escherichia coli* serotype 055:B5; Sigma), rh γ-IFN

(1000 U/ml; Genzyme, Cambridge, MA) or IL-4 (100 ng/ml; a gift from Immunex Corp., Seattle, WA). Cytokines were in M199 medium containing 10% FCS, heparin and endothelial growth factor. In experiments using cycloheximide (Sigma), HUVEC were preincubated for 30 min. with M199 medium containing 10 μg/ml cycloheximide and stimulated in medium containing 10 μg/ml cycloheximide.

*Endothelial-leukocyte attachment assay.* An *in vitro* adhesion assay was developed that is similar to the Stamper and Woodruff frozen section assay (Stamper, et al., J. Exp. Med. 144:828 (1976)) as described (Tedder, et al., J. Immunol. 144:532 (1990)), with some modifications. Endothelial cells were grown to confluence on gelatin (0.1%) coated glass slides, stimulated with TNF-α or other inflammatory agents, washed, and incubated for 15 min. with medium, W6/32, HAE-2 or H18/7 mAb, and washed 4 more times. The mAb reactive with endothelial surface molecules were also added back to the leukocyte suspensions so that the mAb were present throughout the assay. Neutrophils ($5 \times 10^6$) or lymphocytes ($4 \times 10^6$) were suspended in 100 μl RPMI medium containing 5% FCS and mAb(s) or PPME and incubated for 15 min. on ice prior to addition to the endothelial monolayers with rotation at 64 rpm. After 30 min. incubation the medium was tipped off and the slides were placed vertically in fixative (PBS, 1% (v/v) glutaraldehyde, pH 7.4; Polysciences, Warrington, PA) (Tedder et al., J. Immunol. 144:532 (1990)). After overnight fixation, the endothelial cells were stained with hematoxylin and representative fields were photographed (400X, final magnification). The number of adherent leukocytes was determined by counting 4 to 12 microscopic fields (0.09 $mm^2$/field), and the results were expressed as means ± SD. Fields for analysis were located at half radius distances from the center of the endothelial cultures. All endothelial cell monolayers used in these experiments were confluent at the initiation of the assays. Leukocyte binding to the endothelium and mAb inhibition was

uniform except at the center of rotation and at the edge of the endothelial monolayers where some endothelial cell detachment occurred. Treatment of endothelial monolayers with PPME (3 μg/ml) or any of the mAb used in these studies did not alter the integrity of viable HUVEC monolayers. Overnight fixation of confluent endothelial monolayers routinely caused some retraction of the endothelial cells such that the monolayers did not always appear confluent following treatment. However, this slight retraction of the endothelial cells did not alter the number of leukocyt s bound per unit area to a detectable extend when compared with unfixed HUVEC monolayers. In addition, carrying out the assays at 4, 27 or 37°C did not affect the integrity of the endothelial cell monolayers.

In additional experiments, TNF-stimulated (6 hours) endothelial cells were treated with neuraminidase (*Vibrio cholerae*, Calbiochem Corp., San Diego, CA) before the addition of leukocytes. Confluent monolayers of HUVEC on glass slides were fixed with 2% glutaraldehyde for 30 min. at 4°C and rehydrated in PBS. Although treatment of the monolayers with gluteraldehyde before the assays reduced leukocyte binding by ~4 fold, this step was necessary as neuraminidase treatment of unfixed HUVEC at pH 5.5 greatly reduced the integrity of the monolayers. The endothelial cells were then overlaid with 100 μl of neuraminidase (1 U/ml in 50 mM NaOAc, 154 mM NaCl pH 5.5, 4 mM $CaCl_2$) for 30 min. at 37°C. The slides were washed 5 times in RPMI 1640 medium containing 1% FCS and the assays carried out as above at 4°C. Eight fields were examined and counted on each slide.

*Assay for leukocyte attachment to high endothelial venules (HEV) of peripheral lymph nodes.* The HEV-binding assay was performed with frozen tissue sections of rat peripheral lymph nodes using the methods of Stamper and Woodruff (Stamper et al., J. Exp. Med. 144:828 (1976)) and Butcher et al. (Butcher et al., J. Immunol. 134:2989 (1979)) as described (Tedder et al., J. Immunol. 144:532 (1990)).

Briefly, lymphocytes (5 x $10^6$) were treated with neuraminidase (0.005 U/ml) for 30 min. at room temperature, then incubated for 10 min. on ice with the anti-LAM-1 mAbs in medium containing 5% FCS. The cells, in a final volume of 100 µl, were then incubated under rotation (64 rpm) for 30 min. at 4°C on four 12 µm frozen rat peripheral lymph node sections. After fixation overnight in PBS with 1% (w/v) glutaraldehyde, the number of HEVs per tissue section was determined and lymphocyte adhesion to HEV was quantitated.

*Neutrophil transmigration assay.* Neutrophil migration through endothelial cell monolayers was examined using an *in vitro* videomicroscopic transmigration model system that has been described in detail (Luscinskas et al., J. Immunol. 146:1617 (1989)). Briefly, neutrophils (1 ml, 5 x $10^6$) pretreated for 10 min. at 4°C with medium or medium containing mAb were placed on confluent untreated or IL-1ß (10 U/ml) stimulated endothelial monolayers cultured on thick, optically-clear collagen gels that were washed 3 times with RPMI 1640 medium containing 1% FCS. After 20 min. at 37°C, 5% $CO_2$, neutrophil migration across control or IL-1 treated monolayers was assessed using a videomicroscopic system. The data are the percentage of neutrophils that have transmigrated at 20 min.

*Statistical analysis.* Statistical significance of results was determined using the paired Student's t test.

**RESULTS**

*LAM-1 mediates leukocyte binding to activated endothelium.* Anti-LAM-1 mAb were tested for their ability to block lymphocyte and neutrophil binding to cytokine-activated human umbilical vein endothelial cells, using a newly developed assay analogous to the Stamper-Woodruff assay used to assess lymphocyte adhesion to high endothelial venules (HEV) (Stamper et al., J. Exp. Med. 144:828 (1976)). Endothelial cells were grown to confluence on gelatin-coated microscope slides, activated with TNF-α, washed, and th n

overlayered with leukocytes suspended in 100 μl of medium with gentle rotation (64 rpm) of the slides. The assay was carried out at 4°C, where LAM-1 shedding is minimal (Tedder et al., J. Immunol. 144:532 (1990); and Spertini et al., Nature 349:691 (1991)) and CD18-mediated adhesion is inactive (Marlin et al., Cell 68:805 (1991)). Under these conditions, a major proportion of the attachment of both lymphocytes (48 ± 13%) and neutrophils (60 ± 14%) was blocked by the anti-LAM-3 mAb. The treatment of leukocytes and endothelium with mAb reactive with MHC class I antigens and functionally silent epitopes of LAM-1 was without significant effect (~9% inhibition). Thus, neutrophils and lymphocytes both utilize LAM-1 as a major receptor for attachment to activated endothelium.

*LAM-1 is involved in the initial attachment of leukocytes to endothelium*. The involvement of LAM-1 in leukocyte attachment to endothelium was examined with and without rotation of the slides. When the assays were carried out without rotation, anti-LAM1-3 mAb had no significant effect (7 ± 7% inhibition, N=3) on lymphocyte or neutrophil adhesion. In contrast, when the attachment assays were performed using rotating conditions, a significant proportion (56 ± 13%) of lymphocyte ($p<0.025$) and neutrophil adhesion ($p<0.005$) was blocked. The control mAb (anti-LAM1-10) gave results similar to medium alone with and without rotation. Although fewer lymphocytes were bound under rotating conditions compared with static assays, similar numbers of neutrophils were bound under both conditions. These results show that LAM-1 is involved in the initial attachment phase of leukocyte adhesion to endothelium and that rotation may serve to minimize the relative contributions of adhesion molecules which may function in adhesive events subsequent to initial attachment. This is in contrast to results obtained using static adhesion assays at 4°C where most lymphocyte attachment was mediated through CD29 and

neutrophil attachment was mediated through ELAM-1 (Luscinskas et al., J. Immunol. 146:1617 (1991)).

*The same epitopes of LAM-1 mediate lymphocyte attachment to HEV and endothelium*. In earlier studies, mAb that identify spatially and functionally distinct epitopes on LAM-1 (Spertini et al., J. Immunol. (in press) (1991); and Kansas et al., J. Cell Biol. 114:351 (1991)). The role of these epitopes in ligand binding was examined in lymphocyte attachment to endothelium and attachment to rat lymph node HEV. The anti-LAM1-3, -4 and -6 mAb inhibited ~90% of lymphocyte binding to HEV (Spertini et al., Leukemia 5:300 (1991); and Spertini et al., Nature 349:691 (1991)) and inhibited 36-45% of lymphocyte attachment to endothelium. The anti-LAM1-1 and -7 mAb which respectively inhibit 75 and 29% of lymphocyte adhesion to HEV also significantly inhibited lymphocyte adhesion to activated endothelial monolayers. In contrast, four anti-LAM-1 mAb that do not inhibit HEV-binding, or anti-HLA class I mAb, did not inhibit lymphocyte attachment to endothelium. Thus, identical epitopes of LAM-1 mediate binding to endothelium and HEV.

*LAM-1 functions in combination with other adhesion receptors to mediate leukocyte attachment to endothelium*. The contribution of LAM-1 mediated attachment to leukocyte-endothelial adhesion was compared with that of other adhesion proteins at 4, 25 and 37°C. At 4°C, mAb reactive with VCAM-1 on endothelium or the ß chain of its receptor, CD29, on lymphocytes were able to inhibit lymphocyte adhesion by 60 ± 8%. The combination of anti-LAM1-3 and anti-CD29 mAb inhibited ~90% of lymphocyte adhesion. Neutrophil adhesion was inhibited by mAb binding to endothelial ELAM-1 (55 ± 5%), and anti-LAM1-3 mAb in combination with anti-ELAM-1 mAb blocked ~90% of binding. The anti-CD29 mAb had no detectable effect on neutrophil binding to endothelium and the anti-ELAM-1 mAb had no significant effect on lymphocyte binding to endothelium, further demonstrating the specificity of inhibition. CD18 played a minor role in lymphocyte and

neutrophil adhesion at 4°C. Treatment of cells with mAb reactive with functionally silent epitopes of LAM-1 or with anti-class I mAb did not inhibit leukocyte adhesion.

MAb reactive with CD18 inhibited lymphocyte (30%) and neutrophil (39%) adhesion to endothelium at 25°C. Similarly, anti-LAM1-3 mAb inhibited adhesion by 47% for lymphocytes and 46% for neutrophils. The combination of anti-LAM1-3 and anti-CD29 mAb inhibited a major portion of lymphocyte attachment (64%), while the combination of these two mAb with anti-CD18 mAb inhibited 88 to 90% of adhesion. The combination of anti-LAM-1 and anti-ELAM-1 mAb inhibited a significant portion of neutrophil attachment (44%), and the combination of these two mAb with anti-CD18 mAb also inhibited 88 to 90%.

At 37°C, lymphocyte adhesion was significantly inhibited (56%) by anti-LAM1-3 alone ($p<0.05$), while anti-CD18 mAb inhibited by 43%, and anti-CD29 mAb inhibited by 65%. The combination of these mAb inhibited the binding of lymphocytes by ~90%. Neutrophil adhesion was inhibited by anti-LAM-1-3 (27%), CD18 (22%) and anti-ELAM-1 (26%) mAb. Again, combining the anti-LAM-1, anti-ELAM-1 and CD18 mAb blocked ~90% of adhesion. Thus, LAM-1 acts in concert with other adhesion molecules to mediate leukocyte attachment to cytokine activated endothelium, and in this assay system, these receptors in combination can account for nearly all leukocyte attachment.

*Role of LAM-1 in leukocyte transmigration.* Leukocyte entry into sites of inflammation *in vivo* is likely to involve multiple receptor systems. Therefore, the role of LAM-1 in leukocyte extravasation was examined using an *in vitro* videomicroscopic transmigration model system (Luscinskas et al., J. Immunol. 146:1617 (1991)). In this assay, treatment of neutrophils with mAb against HEV-binding epitopes of LAM-1 significantly inhibited transmigration (~30-43%, $p<0.001$), in comparison to treatment with mAb reactive with CD16, functionally silent epitopes of LAM-1, or HLA class I

antigens which were without significant effect. The increase in migration induced by cytokine activation of endothelial monolayers was quantitatively blocked by anti-CD18 or anti-ELAM-1 mAb. Thus, in this functional assay, LAM-1 contributes, in conjunction with other adhesion receptors, to the enhanced neutrophil transmigration observed following cytokine activation of endothelial monolayers.

*Biochemical properties of the endothelial ligand.* PPME is a soluble carbohydrate which specifically inhibits lymphocyte binding to HEV (Yednock et al., J. Cell Biol. 104:713 (1987); Yednock et al., J. Cell Biol. 104:725 (1987); and Stoolman et al., Blood 70:1842 (1987)). Therefore, it was examined for its ability to inhibit leukocyte interactions with endothelium. PPME significantly inhibited ($p<0.001$) lymphocyte and neutrophil attachment to activated endothelium to the same extent as the anti-LAM1-3 mAb. This effect was specific for PPME as another anionic polysaccharide, heparin at 3 μg/ml, did not inhibit lymphocyte or neutrophil attachment to activated endothelium as similarly described for HEV binding (Yednock et al., J. Cell Biol. 104:725 (1987)).

The ligand for LAM-1 on HEV is composed, in part, of sialic acid as neuraminidase treatment of HEV eliminates LAM-1 mediated attachment (Rosen et al., Science 228:1005 (1985); and True et al., J. Cell. Biol. 111:2757 (1990)). Therefore, the role of sialic acid in LAM-1 mediated attachment to activated endothelium was also examined. Fixed endothelial cells bound 8 ± 4 neutrophils per unit area and 4 ± 2 after neuraminidase treatment. TNF-α treatment of the endothelial cells for 6 hours before fixation increased leukocyte binding to 461 ± 117 neutrophils. Treatment of the endothelial cells with neuraminidase reduced binding by 63% to 168 ± 52 neutrophils while treatment of the neutrophils with the LAM1-3 mAb decreased binding by 78% to 102 ± 28. Treatment of endothelium with neuraminidase in conjunction with treatment of neutrophils with anti-LAM1-3 mAb did not increase the

levels of inhibition (66%), resulting in 157 ± 34 neutrophils binding. Therefore, as with HEV, the LAM-1 ligand expressed by activated endothelium appears to contain carbohydrate residues bearing sialic acid as an essential component.

*Inflammatory cytokines induce expression of the LAM-1 ligand by endothelial cells*. Leukocyte attachment to unactivated endothelium at 4°C was minimal, with less than 10 leukocytes bound per field. Stimulation of endothelium with TNF-α induced a dramatic increase in both lymphocyte and neutrophil adhesion to endothelium. IL-1ß or bacterial endotoxin (LPS) also resulted in dramatically increas d lymphocyte adhesion, while treatment with γ-IFN or IL-4 induced smaller, but significant ($p<0.005$), increases. In all cases, treatment of lymphocytes or neutrophils with anti-LAM1-3 mAb blocked attachment by 50 to 70%. It is unlikely that cytokine treatment affected leukocyte function since the endothelium was washed extensively prior to leukocyte binding. Therefore, different inflammatory cytokines and agents were able to induce different levels of leukocyte adhesion mediated through LAM-1.

*Kinetics of endothelial cell expression of the LAM-1 ligand.* Untreated endothelium or endothelium cultured with TNF-α for two hours supported little lymphocyte binding at 4°C. However, a significant increase in lymphocyte adhesion was observed after 4 to 24 hours of TNF-α stimulation. Anti-LAM1-3 specifically inhibited the increased attachment by 36-69%, while mAb against HLA class I antigens or nonfunctional epitopes of LAM-1 were without effect. A similar degree of inhibition of binding was obtained with the anti-CD29 mAb, and the combination of anti-LAM1 and anti-CD29 mAb inhibited most lymphocyte adhesion. Induction of adhesive ligands was dependent on protein synthesis since cyclohexamide (10 μg/ml) treatment of endothelium completely abrogated adhesion. Therefore, expression of the LAM-1 ligand is induced between 2 and 4 hours following endothelial stimulation, and persists for at least 24 hours.

The ability of neutrophils to bind to TNF-stimulated endothelium during this time period was also examined. Although endothelial cells stimulated for 6 hours bound high numbers of neutrophils (800-1000 cells/field), cells stimulated for 24 hours bound only 114 ± 12 neutrophils at 4°C. Stimulated endothelium treated with anti-HLA-class I mAb bound 127 ± 17 neutrophils per field. Treatment of the neutrophils with anti-LAM1-3 mAb reduced binding by 82% (20 ± 5 cells bound) while treatment of the endothelial cells with anti-ELAM-1 mAb blocked binding by 65% (40 ± 11 cells bound). Treatment of the cells with anti-LAM1-3 and anti-ELAM-1 mAb together inhibited neutrophil binding by 95% (5 ± 3). After 36 hours of endothelial cell stimulation, anti-LAM1-3 mAb blocked 75% of neutrophil binding (39 ± 23 cells/field), the anti-ELAM-1 mAb blocked only 21% of binding (118 ± 45), while the anti-LAM1-3 and anti-ELAM-1 mAb together blocked 75% of binding (37 ± 14), in comparison with the anti-LAM1-10 mAb that did not inhibit binding (150 ± 50). These results suggest that at later time points when endothelial cell expression of ELAM-1 and neutrophil adhesion is diminished, LAM-1 contributes relatively more to the interactions of activated endothelium with neutrophils.

Use

Leukocyte migration and infiltration into areas of tissue damage or injury or tissue transplant in a patient can cause or increase pathology. Therefore, methods for detecting sites of damage and agents that impede these infiltration processes can be used for therapeutic treatment. Specifically, leukocyte-mediated inflammation is involved in a number of human clinical manifestations, including th adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response is to image the site of inflammation and to block competitively the adhesive int ractions between leukocytes and the endothelium adjacent to the inflamed

region. On a systemic level, treatment of a patient in shock (e.g., from a serious injury) with an antagonist to cell surface LAM-1 function can result in the reduction of leukocyte migration and adhesion to a level manageable by the target endothelial cells and the subsequent dramatic recovery of the patient.

In addition, subpopulations of malignant cells that express the LAM-1 receptor protein would allow the receptor to function in metastasis of tumor cells. Agents developed to block receptor function can inhibit the metastasis and homing of the malignant cells.

One method of imaging the sites of inflammation in a patient involves detecting the expression of the LAM-1 ligand on the inflamed endothelium. The method includes administering to a patient a pharmaceutical composition consisting of a detectable amount of a labeled LAM-1 protein, or ligand binding fragment of a LAM-1 protein, alone or joined to a carrier protein in a pharmaceutically acceptable carrier. Sufficient time is allowed for the labeled protein to localize at the site of LAM-1 ligand expression, unbound protein is permitted to clear from healthy tissue in the patient, and signal generated by the label is detected and converted into an image of the site of inflammation. The amount labeled LAM-1 protein preferably would be from 1 pg/kg to 10 mg/kg although higher or lower doses are possible depending on the imaging agent label used and the sensitivity of the detection method.

Some of the labels which can be detected externally from within the body of a human patient include radionuclides, radiopaque labels, and paramagnetic isotopes. A radionuclide for *in vivo* diagnosis should have a half-life long enough that it is still detectable at the time of maximum uptake, but short enough that after diagnosis unwanted radiation does not remain in the patient. Coupling of radionuclides to antibodies or proteins is well known in th art (s , e.g., Daddona et al., U.S. Pat. No. 5,026,537, the teachings of

which are incorporated by reference herein) and is often accomplished either directly or indirectly using an intermediary functional group such as a chelating agent. Examples of radioisotopes that could be used for *in vivo* diagnosis are $^{99}Tc$, $^{123}I$, $^{131}I$, $^{111}In$, $^{97}Ru$, $^{97}Cu$, $^{67}Ga$, $^{68}Ga$, $^{72}As$, $^{89}Zr$, and $^{201}Ti$. Paramagnetic isotopes for purposes of *in vivo* diagnosis can also be used according to the methods of this invention. Examples of elements that are particularly useful for use in magnetic resonance energy techniques include $^{157}Gd$, $^{55}Mn$, $^{162}Dy$, $^{52}Cr$, and $^{56}Fe$. For radiopaque imaging, the LAM-1 protein or ligand binding fragment is coupled to an agent that produces an opaque field at the site of inflammation upon X-ray imaging.

Individual domains of LAM-1, ligand binding fragments of LAM-1, or the entire LAM-1 protein can then be used for therapeutic treatment to interfere in the binding of leukocytes (expressing the LAM-1 protein) at the site of inflammation. In addition, the LAM-1 protein, a specific domain, or a ligand binding fragment can be joined to a carrier protein to increase the serum half-life of the therapeutic agent. For example, a LAM-1 fusion protein with human IgG1 heavy chain, an antibody-like immunoglobulin chimera, was produced as follows: an altered fragment of LAM-1 cDNA was produced that generated a *Ban* II endonuclease cleavage site within the exon VIII encoded domain by PCR using an antisense primer with the sequence GTTATAATCGGGCTCCTTAATC. This generated a *Ban* II sequence at nucleotide positions 1073-1078 (Fig. 2). The LAM-1 cDNA fragment encoding the leader, lectin, EGF-like and SCR domains plus this altered spacer domain were fused at this *Ban* II site to the *Ban* II site within the hinge region of a human IgG1 heavy chain cDNA. This generated a cDNA encoding the LAM-1 extracellular domains fused with the hinge, CH2, and CH3 domains of IgG1. This cDNA was subcloned into a mammalian expression vector and was subsequently transfected into COS-7 cells. The soluble dimeric LAM-1/IgG1 protein

product secreted into the medium retained all of the antigenic epitopes of the LAM-1 molecule defined by 16 anti-LAM-1 monoclonal antibodies. In a similar fashion a soluble immunoglobulin chimera can be obtained for each specific exon-encoded domain of LAM-1, or fragment thereof. The immunoglobulin chimera are easily purified through IgG-binding protein A-Sepharose chromatography. The chimera have the ability to form an immunoglobulin-like dimer with the concomitant higher avidity and serum half-life.

Other agents can also be joined to the LAM-1 protein or to a specific domain or ligand binding fragment to form a useful product. For example, the LAM-1 lectin domain can be combined with the toxic portion of a cytotoxin to produce a fusion protein. In addition, the LAM-1 protein or protein portions may be coupled to a chemotherapeutic drug or drugs which could bind to cells expressing the LAM-1 ligand, to administer the drug to a site of tissue damage or inflammation to treat, e.g., acute inflammation or vasculitus. Such drugs may include, anti-inflammatory agents or agents that provide regional relief from inflammatory distress. Syndromes, diseases, and conditions that could be treated by these agents would include, but not be limited to, treating inflammation, microbial/parasitic infections, post-reperfusion injury, leukemia, lymphoma, vasculitis, inhibition of the metastatic spread of tumor cells, organ transplantation, or graft rejection. The fusion proteins can be transcribed from a cDNA hybrid molecule, as described above, or the agent may be covalently bonded to the LAM-1 protein or domain by routine procedures.

A ligand binding fragment of the LAM-1 protein can be experimentally determined using this invention. In a typical procedure, fragments of the LAM-1 cDNA can be fused with cDNA encoding a carrier protein, such as immunoglobulin heavy chain on the CD62 cDNA as described by Kansas et al. (J. Cell Biol. 114:351 (1991) hereby incorporated by reference herein). These chimeric proteins can be expressed on the

cell surface, or as soluble molecules, and their ability to bind ligand assessed. For example, the ability of LAM-1 fusion proteins to bind PPME or fucoidin requires only the lectin domain of LAM-1 (Kansas et al., supra). Further segregation of fragments of the LAM-1 protein or peptides from the LAM-1 protein can be examined for their ability to bind ligands, as well as their ability to inhibit the binding of LAM-1 protein to ligand.

A method for detecting LAM-1 ligand expression in a biological sample uses the LAM-1/ligand binding reaction and comprises contacting the sample with the LAM-1 protein or LAM-1$^+$ cells, and detecting whether a complex forms. This invention allows the detection and biochemical identification of the LAM-1 ligand. First, the ligand can be identified directly by the production of monoclonal antibodies reactive with molecules expressed on activated endothelium that are able to inhibit the attachment of leukocytes to the endothelial cells through LAM-1. These blocking antibodies can be produced and tested by routine procedures and used to identify the endothelial ligand and its tissue distribution and to isolate and purify the ligand for biochemical analysis and amino acid sequence determination. Clones of cDNA and genomic DNA encoding the LAM-1 ligand can also be isolated by standard procedures and used to synthesize the ligand for therapeutic utility.

In addition, the use of this assay will allow the development of antagonists which can inhibit the function of LAM-1. We have already developed one class of antagonists composed of monoclonal antibodies that bind to ligand-binding epitopes on LAM-1 and thus inhibit LAM-1 function. Alternatively, LAM-1 fragments or ligand binding-domains fused with other proteins can be tested for their ability to inhibit the binding of leukocyt s to endothelium through LAM-1. For example, antagonists have been developed which are composed of the LAM-1 extracellular domains fused with the hinge, CD2 and CD3 domains of immunoglobulin heavy chain.

These agents inhibit LAM-1 mediated attachment of leukocytes. Another similar construct composed of LAM-1 fragments that has been examined using this invention is a fusion protein composed of the ligand binding domain of LAM-1 fused with the EGF-like and short consensus repeat domains of CD62 bound to a cell surface. In a similar fashion the ligand binding domain of LAM-1 can be substituted for the ligand binding domain of another selection such as ELAM-1 or GMP-140 to form an antagonistic construct. This invention will allow the development of pharmacologic reagents composed of peptides, carbohydrate moieties, RNAs or other small molecules which may mimic the LAM-1 ligand or ligand-binding epitopes of LAM-1, and inhibit the attachment of leukocytes to endothelium through this receptor.

In another therapeutic method the LAM-1 protein or ligand binding fragment, or the LAM-1 ligand or fragment thereof can be used in combination therapy with any other selectin or any other cell surface molecule, or soluble fragments thereof, involved in adhesion of leukocytes to endothelial surfaces. In addition, antagonists to any of the above receptors or receptor portions (or mAb reactive to the receptors or receptor portions) can be used in the above combinations or as independent antagonist combinations for treatment of a patient. Examples include ICAM-1, VCAM-1, VLA-4, CD18, CD11a, CD11b, CD31, GMP-140, and ELAM-1 and the mAb reactive with them.

In addition, other tissues can be examined for the presence of the endothelial LAM-1 ligand. For instance, following reperfusion injury, vascular endothelium in multiple organs may express the LAM-1 ligand. Furthermore, soluble (non-cell bound) LAM-1 ligand-binding agents that are labeled directly or detected by antibodies reactive with the LAM-1 fragment can be used to detect and quantify LAM-1 ligand in biological fluids, on a cell surface, or bound to a solid matrix such as nitrocellulose. For example, a tissue biopsy sample can be reacted with LAM-1 or a ligand-binding

fragment thereof, and the binding of LAM-1 monitored using standard immunological or immunohistological assays. The reactivity of LAM-1 with this tissue would be a measure of inflammation and thereby a prognostic indicator, such as in the case of transplanted organ rejection. Alternately, the LAM-1 ligand can be isolated from endothelium by forming a LAM-1 protein/ligand complex in a sample, which may then be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for LAM-1 or a carrier protein containing a ligand binding fragment of LAM-1. The isolated LAM-1 ligand can be biochemically identified and purified by standard techniques or the LAM-1/ligand complex can be quantitated to give a measure of endogenous LAM-1 ligand and, thereby, the extent of stimulation of endothelial cells. Of course, the specific concentrations of detectably labeled LAM-1 or ligand-binding fragment thereof and the temperature and time of incubations, as well as other assay conditions, may be varied depending on various factors including the concentration of LAM-1 ligand in the sample. These parameters can be determined by those skilled in the art.

Moreover, the LAM-1 ligand may be shed from the cell surface following activation of the endothelium during various inflammatory reactions. The LAM-1 ligand assay or other standard immunological assays (e.g., an ELISA assay) using LAM-1, or ligand-binding fragments thereof, may be used to assess the extent of LAM-1 ligand shedding and to permit monitoring of the progress of an inflammatory event, microbial infection, vasculitis or organ transplant rejection by the presence of a LAM-1 binding component of the ligand in body fluids. Typical fluids that can be monitored are serum, plasma, cerebrospinal fluid, urine, saliva, semen, lymph or other secretions.

The therapeutic agents may be administered orally, topically, or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, or intra-

arterially) by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosage and modes of administration can readily be determined by conventional protocols.

The *lyam-1* gene itself can also be used in genetic therapy. Individuals having a genetic defect in the *lyam-1* gene would be unable to produce a fully active LAM-1 protein leukocyte "homing" receptor and thus would be unable to mobilize sufficient leukocytes to a site of inflammation. Individuals suspected to having a congenital defect in the *lyam-1* gene could be screened for this genetic disorder using the sequence and structural information described. Treatment of affected individuals would then be possible using the *lyam-1* gene or fragments thereof.

The normal regulation of the *lyam-1* gene, as evidenced by the appearance and disappearance of the LAM-1 protein on the surface of a specific leukocyte sub-population can be monitored to test the effects of drugs or specific therapies that would alter gene expression.

Other embodiments are within the following claims.

CLAIMS

What is claimed is:

1. A method of detecting a site of inflammation or a diseased state in a human patient comprising

administering to said patient a pharmaceutical composition comprising a detectable amount of a labeled LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance, and

detecting the label on said LAM-1 protein.

2. The method of claim 1 wherein said LAM-1 protein or ligand binding fragment thereof is labeled with any one of a radionuclide, a paramagnetic isotope, or a radiopaque label.

3. The method of claim 1 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a carrier protein.

4. The method of claim 3 wherein said carrier protein comprises an immunoglobulin heavy chain constant region.

5. The method of claim 3 wherein said carrier protein comprises non-ligand binding portions of a selectin molecule.

6. An imaging agent for imaging a site of inflammation or a diseased state in a human patient comprising

LAM-1 protein or a ligand binding fragment thereof labeled with a detectable label and suspended in a pharmaceutically acceptable carrier substance.

7. The imaging agent of claim 6 wherein said detectable label comprises any one of a radionuclide, a paramagnetic isotope, or a radiopaque label.

8. The imaging agent of claim 6 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a carrier protein.

9. The imaging agent of claim 9 wherein said carrier protein comprises an immunoglobulin heavy chain constant region.

10. The imaging agent of claim 9 wherein said carrier protein comprises non-ligand binding portions of a selectin molecule.

11. A method of treating a human patient suffering from a leukocyte-mobilizing condition comprising administering to said patient a therapeutic composition comprising a therapeutic amount of LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance.

12. The method of claim 11 wherein said patient suffers from tissue damage or an autoimmune disorder.

13. The method of claim 11 wherein said patient suffers from cancer.

14. The method of claim 11 wherein said patient is an organ or tissue transplant recipient.

15. The method of claim 11 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a therapeutic agent.

16. The method of claim 15 wherein said therapeutic agent comprises either a chemotherapeutic drug or an anti-inflammatory agent.

17. A method of treating a human patient suffering from a leukocyte-mobilizing condition comprising administering to said patient a therapeutic composition comprising a therapeutic amount of an antagonist to LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance.

18. The method of claim 17 wherein said patient suffers from tissue damage or an autoimmune disorder.

19. The method of claim 17 wherein said patient suffers from cancer.

20. The method of claim 17 wherein said patient is an organ or tissue transplant recipient.

21. The use of LAM-1 protein or ligand binding fragment thereof to identify a ligand that binds to said protein.

22. The use of LAM-1 protein or ligand binding fragment thereof to identify a ligand that binds to a molecule specifically associated with said protein, or a fragment thereof.

23. The method of claim 17 wherein said antagonist comprises a ligand to LAM-1 protein, or a portion thereof.

24. The method of claim 11 or claim 17 further comprising administering to said patient a therapeutic amount of a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or soluble fragment of said cell surface molecule, said cell surface molecules or soluble fragments being capable of participating in the adhesion of leukocytes to endothelial surfaces.

25. The method of claim 11 or claim 17 further comprising administering to said patient a therapeutic amount of an antagonist to a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or soluble fragment of said cell surface molecule, said cell surface molecules or soluble fragments being capable of participating in the adhesion of leukocytes to endothelial surfaces.

26. A method of determining the extent of binding of blood leukocytes to activated human endothelium comprising the steps of

providing a monolayer of human endothelial cells grown to confluence on a solid support,

activating said confluent endothelial cells,

rotating the endothelial cells on the solid support in said suspension of leukocytes,

removing said suspension, and

determining the number of leukocytes adhering to said endothelial cells.

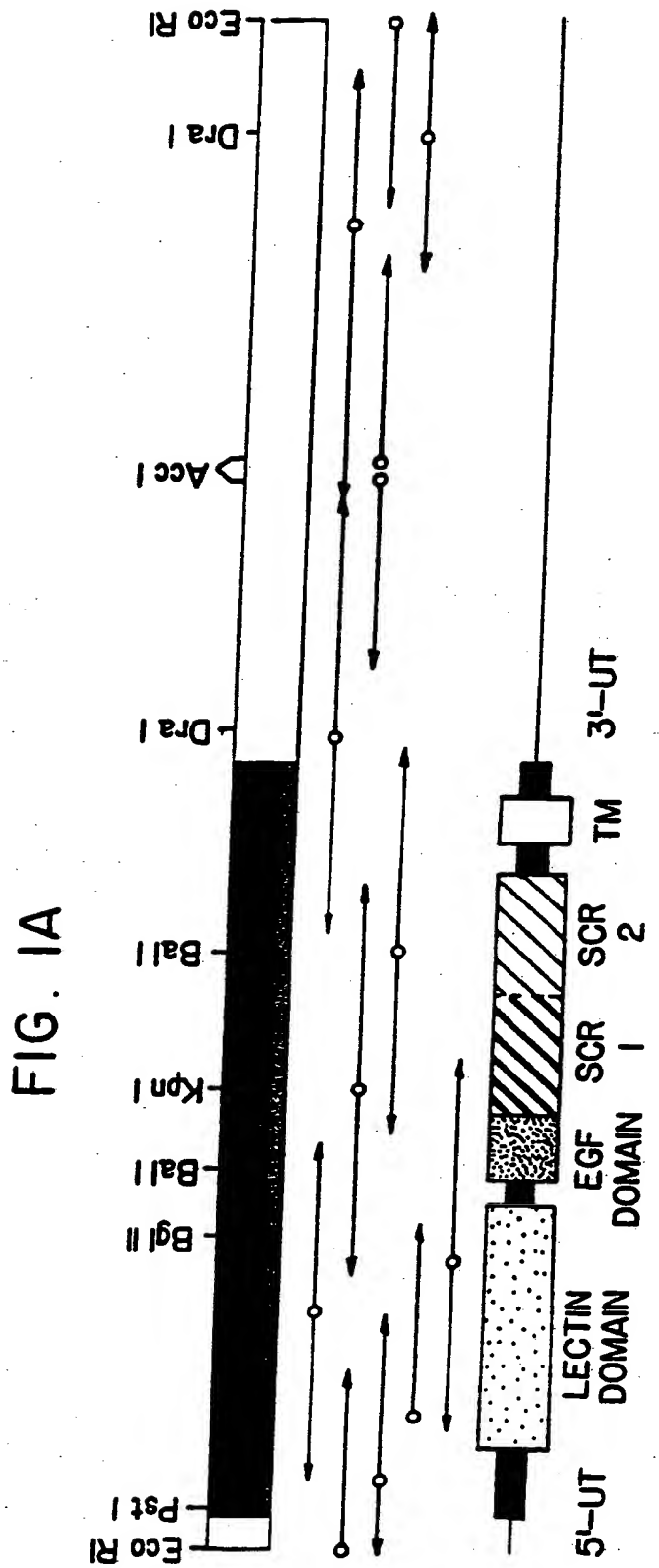


FIG. IA

FIG. IB

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                                                                1
                                                                M   G
gaattcCCTTT GGGCAAGGAC CTGAGACCCT TGTGCTAAGTCAAGAGGCTCA ATG GGC
                    10
  C   R   R   T   R   E   G   P   S   K   A   M
TGC AGA AGA ACT AGA GAA GGA CCA AGC AAA GCC ATG    94
                    20                              30
  I   F   P   W   K   C   Q   S   T   Q   R   D   L   W   N   I
ATA TTT CCA TGG AAA TGT CAG AGC ACC CAG AGG GAC TTA TGG AAC ATC
                    40
  F   K   L   W   G   W   T   M   L   C   C   D
TTC AAG TTG TGG GGG TGG ACA ATG CTC TGT TGT GAT   168
                    50                  ↓
  F   L   A   H   H   G   T   D   C   W   T   Y   H   Y   S   E
TTC CTG GCA CAT CAT GGA ACC GAC TGC TGG ACT TAC CAT TAT TCT GAA
        60
  K   P   M   N   W   Q   R   A   R   R   R   F   C
AAA CCC ATG AAC TGG CAA AGG GCT AGA AGA TTC TGC   262
                    80
  R   D  [N   Y   T]  D   L   V   A   I   Q   N   K   A   E   I
CGA GAC [AAT TAC ACA] GAT TTA GTT GCC ATA CAA AAC AAG GCG GAA ATT
            90
  E   Y   L   E   K   T   L   P   F   S   R   S
GAG TAT CTG GAG AAG ACT CTG CCT TTC AGT CGT TCT   346
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FIG. 2A

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      100                                                   110
  Y   Y   W   I   G   I   R   K   I   G   G   I   W   T   W
 TAC TAC TGG ATA GGA ATC CGG AAG ATA GGA GGA ATA TGG ACG TGG
                             120
  V   G   T  [N   K   S]  L   T   E   E   A   E   N
 GTG GGA ACC [AAC AAA TCT] CTC ACT GAA GAA GCA GAG AAC  430
         130                                          140
  W   G   D   G   E   P   N   N   K   K   N   K   E   D   C
 TGG GGA GAT GGT GAG CCC AAC AAC AAG AAG AAC AAG GAG GAC TGC
                                 150
  V   E   I   Y   I   K   R   N   K   D   A   G   K
 GTG GAG ATC TAT ATC AAG AGA AAC AAA GAT GCA GGC AAA  514
                     160
  W   N   D   D   A   C   H   K   L   K   A   A   L   C   Y
 TGG AAC GAT GAC GCC TGC CAC AAA CTA AAG GCA GCC CTC TGT TAC
170                                         180
  T   A   S   C   Q   P   W   S   C   S   G   H   G
 ACA GCT TCT TGC CAG CCC TGG TCA TGC AGT GGC CAT GGA  598
                         190
  E   C   V   E   I   I   N  [N   Y   T]  C   N   C   D   V
 GAA TGT GTA GAA ATC ATC AAT [AAT TAC ACC] TGC AAC TGT GAT GTG
         200                                         210
  G   Y   Y   G   P   Q   C   Q   F   V   I   Q   C
 GGG TAC TAT GGG CCC CAG TGT CAG TTT GTG ATT CAG TGT  682
                             220
  E   P   L   E   A   P   E   L   G   T   M   D   C   T   H
 GAG CCT TTG GAG GCC CCA GAG CTG GGT ACC ATG GAC TGT ACT CAC
                 230
  P   L   G   N   F   N   F   N   S   Q   C   A   F
 CCT TTG GGA AAC TTC AAC TTC AAC TCA CAG TGT GCC TTC  766
     240                                         250
  S   C   S   E   G   T  [N   L   T]  G   I   E   E   T   T
 AGC TGC TCT GAA GGA ACA [AAC TTA ACT] GGG ATT GAA GAA ACC ACC
                         260
  C   E   P   F   G  [N   W   S]  S   P   E   P   T
 TGT GAA CCA TTT GGA [AAC TGG TCA] TCT CCA GAA CCA ACC  850
             270                                   280
  C   Q   V   I   Q   C   E   P   L   S   A   P   D   L   G
 TGT CAA GTG ATT CAG TGT GAG CCT CTA TCA GCA CCA GAT TTG GGG
                             290
  I   M  [N   C   S]  H   P   L   A   S   F   S   F
 ATC ATG [AAC TGT AGC] CAT CCC CTG GCC AGC TTC AGC TTT  934
                     300
  T   S   A   C   T   F   I   C   S   E   G   T   E   L   I
 ACC TCT GCA TGT ACC TTC ATC TGC TCA GAA GGA ACT GAG TTA ATT
310                                         320
  G   K   K   K   T   I   C   E   S   S   G   I   W
 GGG AAG AAG AAA ACC ATT TGT GAA TCA TCT GGA ATC TGG 1018
                             330
  S  [N   P   S]  P   I   C   Q   K   L   D   K   S   F   S
 TCA [AAT CCT AGT] CCA ATA TGT CAA AAA TTG GAC AAA AGT TTC TCA
         340                                         350
  M   I   K   E   G   D   Y   N   P   L   F   I   P
 ATG ATT AAG GAG GGT GAT TAT AAC CCC CTC TTC ATT CCA 1102
```

FIG. 2B

```
                                                    360
 V   A   V   M   V   T   A   F   S   G   L   A   F   I   I
GTG GCA GTC ATG GTT ACT GCA TTC TCT GGG TTG GCA TTT ATC ATT
                    370
 W   L   A   R   R   L   K   K   G   K   K   S   K
TGG CTG GCA AGG AGA TTA AAA AAA GGC AAG AAA TCC AAG 1186
    380
 R   S   M   N   D   P   Y   *
AGA AGT ATG AAT GAC CCA TAT TAA ATCGCCCTTG GTGAAAGAAA

ATTCTTGGAA TACTAAAAAT CATGAGATCC TTTAAATCCT TCCATGAAAC 1280

GTTTTGTGTG GTGGCACCTC CTACGTCAAA CATGAAGTGT GTTTCCTTCA

GTGCATCTGG GAAGATTTCT ACCTGACCAA GAGTTCCTTC AGCTTCCATT 1380

TCACCCCTCA TTTATCCCTC AACCCCCAGC CCACAGGTCT TTATACAGCT

CAGCTTTTTC TCTTTTCTGA GGAGAAACAA ATAACACCAT AAAGGGAAAG 1480

GATTCATGTG GAATATAAAG ATGGCTGACT TTGCTCTTTC TTGACTCTTG

TTTTCAGTTT CAATTCAGTG CTGTACTTGA TGACAGACAC TTCTAAATGA 1580

AGTGCAAATT TGATACATAT GTGAATATGG ACTCAGTTTT CTTGCAGATC

AAATTTCGCG TCGTCTTCTG TATACGTCCA GGTACACTCT ATGAAGTCAA 1680

AAGTCTACGC TCTCCTTTCT TTCTAACTCC AGTGAAGTAA TGGGGTCCTG

CTCAAGTTGA AAGAGTCCTA TTTGCACTGT AGCCTCGCCG TCTGTGAATT 1780

GGACCATCCT ATTTAACTGG CTTCAGCCTC CCCACCTTCT TCAGCCACCT

CTCTTTTTCA GTTGGCTGAC TTCCACACCT AGCATCTCAT GAGTGCCAAG 1880

CAAAAGGAGA GAAGAGAGAA ATAGCCTCCG CTGTTTTTTA GTTTGGGGGT

TTTGCTGTTT CCTTTTATGA GACCCATTCC TATTTCTTAT AGTCAATGTT 1980

TCTTTTATCA CGATATTATT AGTAAGAAAA CATCACTGAA ATGCTAGCTG

CAACTGACAT CTCTTTGATG TCATATGGAA GAGTTAAAAC AGGTGGAGAA 2080

ATTCCTTGAT TCACAATGAA ATGCTCTCCT TTCCCCTGCC CCCAGACCTT

TTATCCACTT ACCTAGATTC TACATATTCT TTAAATTTCA TCTCAGGCCT 2180

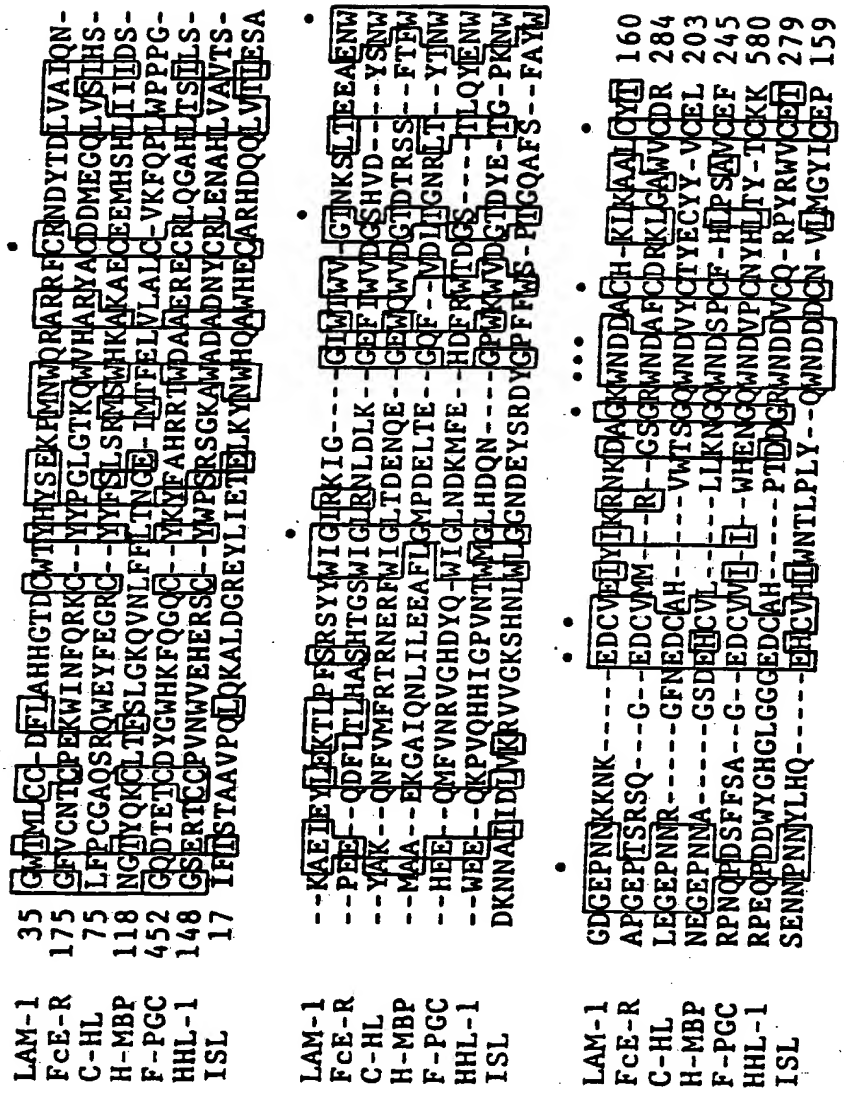
CCCTCAACCC CACCACTTCT TTTATAACTA GTCCTTTACT AATCCAACCC

ATGATGAGCT CCTCTTCCTG GCTTCTTACT GAAAGGTTAC CCTGTAACAT 2280

GCAATTTTGC ATTTGAATAA AGCCTGCTTT TTAAGTGTTA AAAAgaattc 2330
```

FIG. 2C

FIG. 3A



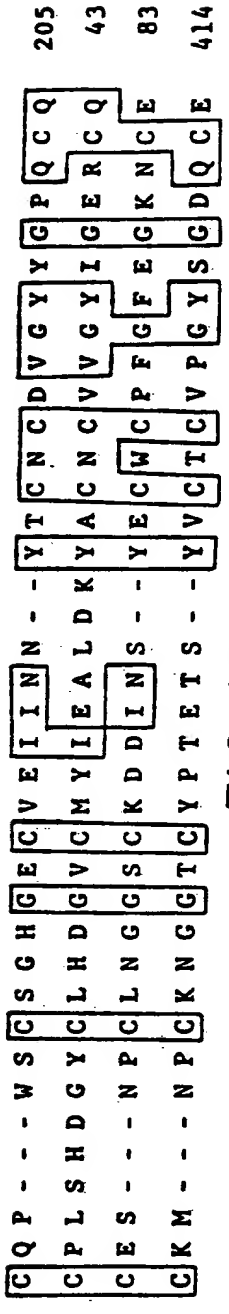



FIG. 3B

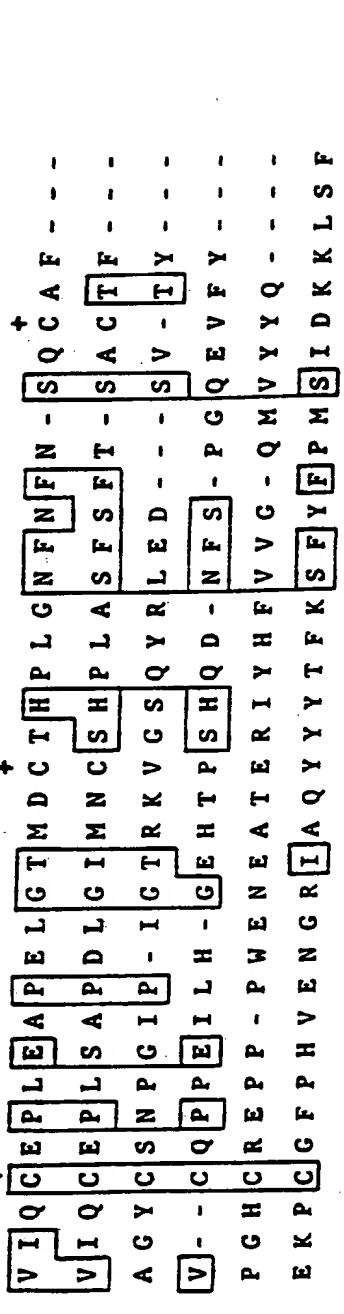
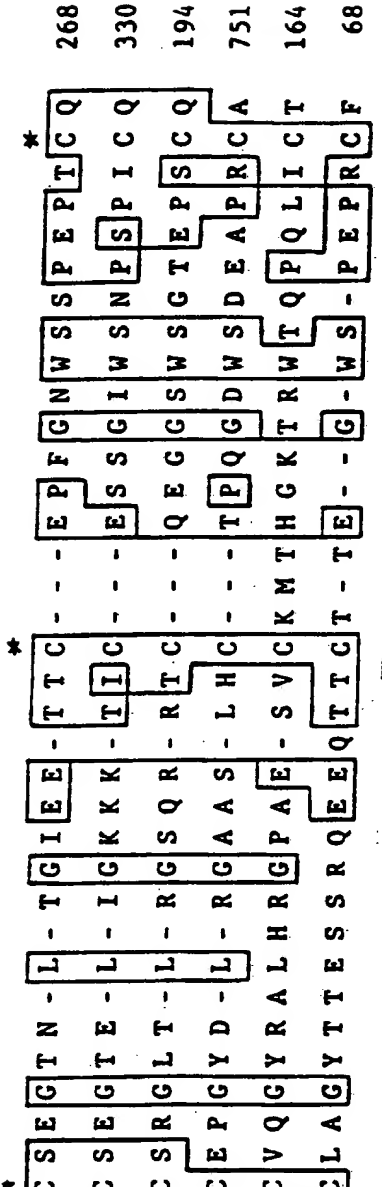



FIG. 3C



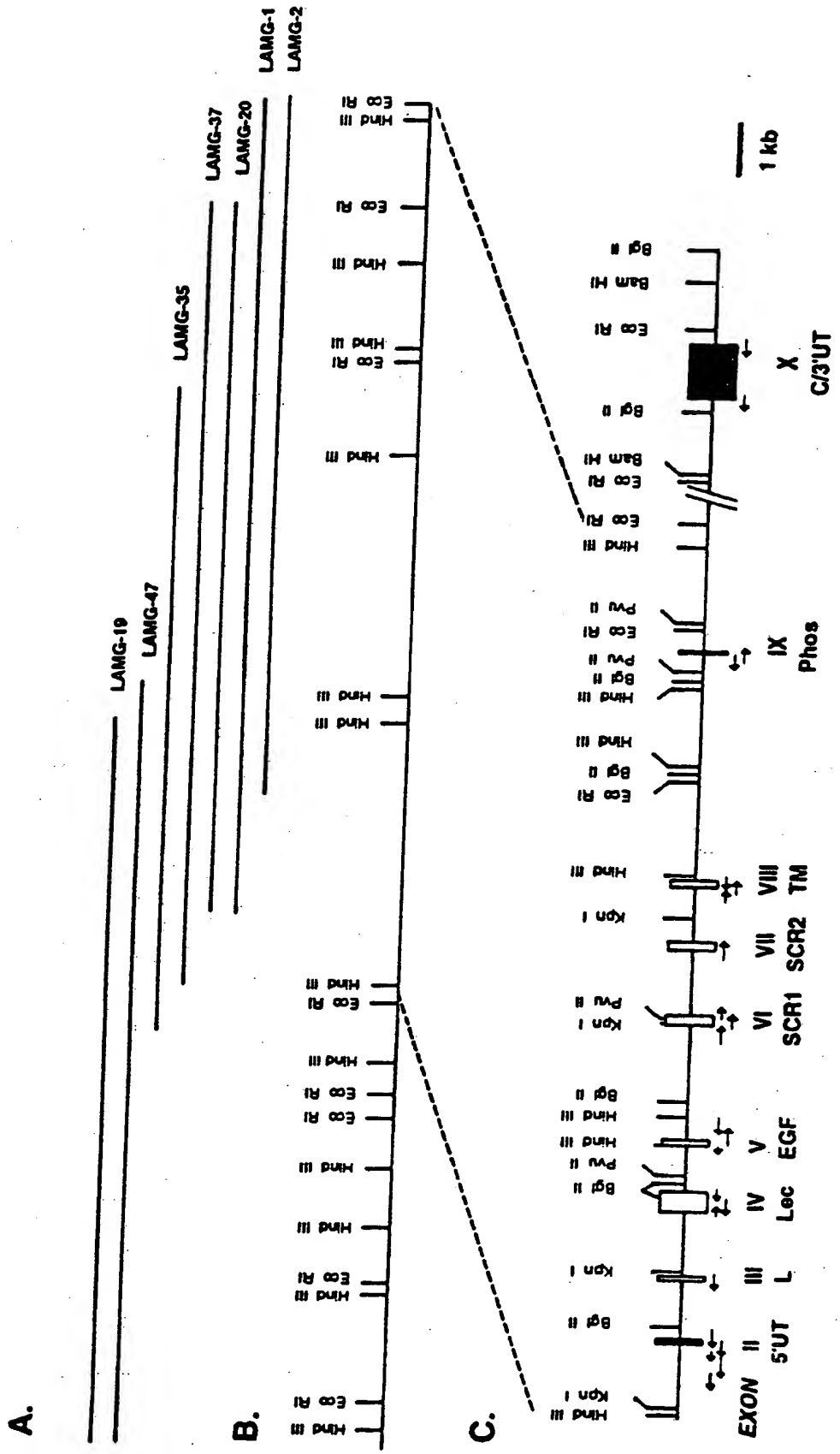



FIG. 4

EXON II (5' Untranslated Region and Translation Initiation Site)

AGAGAGCTG TTATAAAGAT TAAACAATAT AATAAATATG GCGCGTGAGC TTCAGAGTTT TTGTTGTTGT TATTATTATT TTCCTAAAAA TGCAAATCTG ATTTGCATTT
GACTCATTGA CTCACATCAG TGGGTCTTCC TTTTTATTGT CCTTCATCAT ATGGGTCCTA ATTTCACATG CAGTCTTATA AAACCATCTC ATTTTATAGT CCAAGAATAT
TAAAGGTACT TGTAGGCTCC CAAACCTACA CGGTGAAAAG CTAGAGAGCA TGGGCTCTCT TCAGGGGTTA ACTTCAGGAA GTGCCACTAA CAAGGACGTC CACTAGGTGG
TGAGCAAGGA AAGACGGAGG TGAAGGAACC GAAACGAGTC AAGTCCACTG CTTAGCTCTA CTGAAGTTTT GCAAACATCA TAAATATGTC TGAAATGCAG TTTTGATTTG
TGAAGGAGGA AGAGGAGAAC AAGAACCAAA CAAAAACAAG AACAAGAACA AGTAGAAGAA GAGGAGCAGG GAGGAAAAAG AAGAGGAAGA AGAACAGCAA CAACAATGAG
TGAAGGAGGA GGAGGGTAAG GAAAGATGCA TAGGAGAATG GAAGGAAGGA TAGAAAGGAG GGAAGGAAGA GAGAATCTAG TCACATTACT TTCTGATCAG CAGTTCATTT
TTGTCTCAGT GGGAGGCAAT AGAGGCCAGT CTAGGAAAGG GGTGGGGAAA GAGGAAAGAG AAGTGCAGGA GGAAGGGGAG GCCCAAGGGG AGGAGGAGGA GGATGTGAGA
CAAGAGGCTC AATGGGCTGC AGAAGAACTA GAGAAGGACC AAGCAAAGCC ATG GTGAGCCTTT CAGCCTAAAA GACGTTTAGA TGCTCAGATA GAAACTCTTG
GGGTTGTAGA GGCAGGTGGC AAGGATAGGA ATCACCCCAT TTCAATTCTG GTTTTAAATA ATATAGAAAC TAAACATTTT CTCAGACCCT CAAAAAAAGT

EXON III (Leader Domain)

CACTGAGA CTAAGCGTAA AATAAATAGA ACAAACAAAC TGTGCATCAG TTCTGATGTA AATTTGAAGT AATTTTCATC TATGTCTGAG AAACCTGTTA CCTCAGACAG
GGTTAGTAGA CATATGTGTT TTATTCTGAT TATTAAGAAA GTTGTAAGCA CCACCTCAAA GGCTATAAAT GTGTGGTTTA AGGGTATACA TCTAAATATA ATTTTGTATT
TCATTTGCAG ATA TTT CCA TGG AAA TGT CAG AGC ACC CAG AGG GAC TTA TGG AAC ATC TTC AAG TTG TGG GGG TGG ACA ATG CTC TGT
TGT G GTATGTTATG ATATTTATAT ATCACTAAGT CTATTTTACT TATATTCATT TTT

FIG. 5A

EXON IV (Lectin Domain)

CT GGAGTAGTGC TAGGTTCTTT TTAGCTGTAA CATTATGTAA GTCTGCATAG GTCACACTGA TGTCTTGCAG AT TTC CTG GCA CAT CAT GGA ACC GAC TGC
TGG ACT TAC CAT TAT TCT GAA AAA CCC ATG AAC TGG CAA AGG GCT AGA AGA TTC TGC CGA GAC AAT TAC ACA GAT TTA GTT GCC ATA
CAA AAC AAG GCG GAA ATT GAG TAT CTG GAG AAG ACT CTG CCT TTC AGT CGT TCT TAC TAC TGG ATA GGA ATC CGG AAG ATA GGA GGA
ATA TGG ACG TGG GTG GGA ACC AAC AAA TCT CTC ACT GAA GAA GCA GAG AAC TGG GGA GAT GGT GAG CCC AAC AAC AAG AAG AAC AAG
GAG GAC TGC GTG GAG ATC TAT ATC AAG AGA AAC AAA GAT GCA GGC AAA TGG AAC GAT GAC GCC TGC CAC AAA CTA AAG GCA GCC CTC
TGT TAC ACA G GTAGGGAGTG ACAAGACGGC TATGCTGCCT CAGACTCAGG AAGGGCCACG GTTAAGAGAA TACTCAGATT TA

EXON V (EGF Domain)

AAAATTTTAG CCATATGATT TTTATGCTAT GAATTTACCA AATAAACCTT TCCTGATTAT TTAAATCATC TCAGACAAAA GGTTATCTAT GTCTAAAGAA ATGACTTTGA
GTACTAAAAT GTAATCACAT TAAAATATTT TTTTTCTGAC CTCCTTAAAG CT TCT TGC CAG CCC TGG TCA TGC AGT GGC CAT GGA GAA TGT GTA
GAA ATC ATC AAT AAT TAC ACC TGC AAC TGT GAT GTG GGG TAC TAT GGG CCC CAG TGT CAG TTT G GTAAGTCTCT TTCCTTTCTT
TGCTTCTTCT TAGGTAAAGT CACAGGAATC ATTATAGCTT ATCATGAAGC TGGTTGGAAC AAAATGATAC TAGCCACTCT GAGAAATGGG AAGTTTTGAT CAGAAAGCTC
TGCTTTCACA ATATTGTTAC CTTTCCGTAA AGATTTCATA AGTCAGCATG AAGTTTCGAT TCACTTCTCA ACAAGTCTTT TTGAGTACCA CAAGAAGCAC AGTGTTGGGA
TAAAGCTGTC AGGGTTACAA TAAGGAATTA GCATGGTAGA TTCCCGCTCT CAAGAAGCTC ACGATCTAAT GAGCTTGTTA GATTAATTAG AACTCTAAGG TCTGGAAGAA
ACTATGCCAT TTATCATTAG GAGGCTGAGT TACCCAGAAA GTATCTTGCT TTTTCCTTCT AGTAGTTCCT TTCCTTCTTG CAGTTCTCCA CACTTAACAC ATGTGCTCTG
TAGCACACTG ACTTTGCTGG TGGCCTTCTC TCTCATTTTG CACATGGCCA AAAAACATGT CATCTTTAAG ACATTGTTCA AAGACAGTTT CTTCTAGGAA GCTT

EXON VI (SCR 1 Domain)

CTCTGA TGTGATAGTT ATTTCCCGAC TAAGCTGGTC ATTCCCAGTT ACACCTATTT GGCTTTAAGG ATTCTCACTA CAGATAATAC TGAAGATAAT AATATGAAGA
CTAGCTAATG TTTACTTAGA ATTTCTGATG AGTCAGGCTT TGTTCTAACG TCCTTGACTT ATGCTAATTG AATTACATTT AGTTTCCATA TCAATTTGAT AAAGATAACA
CAATTTCATT ATTCCTCTTA TATAGATGAA GAAACTGAAG TTGGAGGGTT CAAGTAACCT TGTTTAAAGG CACATGGTTA TCAAGTGGCA GGGCTAGGAT TCAAATCCAG
GCGTCAGTTC CTCTTAACTC TTCCCCATAC TGTTTCTTTC CCTATTGAAG TG ATT CAG TGT GAG CCT TTG GAG GCC CCA GAG CTG GGT ACC ATG GAC
TGT ACT CAC CCT TTG GGA AAC TTC AGC TTC AGC TCA CAG TGT GCC TTC AGC TGC TCT GAA GGA ACA AAC TTA ACT GGG ATT GAA
GAA ACC ACC TGT GGA CCA TTT GGA AAC TGG TCA TCT CCA GAA CCA ACC TGT CAA G GTGAGTAACT TCAGACTAGA GGTTTTGTCA TGCAATCCTG
GGCTTACAGT CAGAACATTC AGTAGAAGTT TGCTGAGAAG TCAAACTTAG GATCCTAATT TAACCTAACT TTTGTTTAAC CTACTGTGAT GTTTCTCAAA GGACTTATTC

FIG. 5B

EXON VII (SCR II Domain)

GAGGGTCAC CTTAGCTAGG GCAGCAGCCT GGAGTAGCTA CTCCTCTCCC CACAGCTTTC AATGCTTCCT TGCCTTCATC TCTCATTCAC CACCCACCAT CATTCTCAAG
AAAATAAAGC CTGGAAGCAA TATCACAAGT AATGTAGTCA GGCAGCTTTG GCTAAAAATC CAAAGCTCAA GGGAGGGTCT CTACTCAGAA ATACTGTTTT GTCTTTTTTT
TTTTTTCTTT TTCATTGAAG IG AII CAG IGI GAG CCI CIA ICA GCA CCA GAI IIG GGG AIC AIG AAC IGI AGC CAI CCC CIG GCC AGC
IIC AGC III ACC ICI GCA IGI ACC IIC AIC IGC ICA GAA GGA ACI GAG IIA AII GGG AAG AAG AAA ACC AII IGI GAA ICA ICI
GGA AIC IGG ICA AAI CCI AGI CCA AIA IGI CAA A GTGAGTAAGT TTGTCCTGGA ACTGAA

EXON VIII (Transmembrane Domain)

TATCAGAAC TAAGAAAGCT TGGGCTGCAG GTCGACTCTA GGTGCATTTT CAGGAACTCT ATGAACCACA AATCTGGGCA TTGAGATTCT GTAGGCATTA GACTAGCAAG
GCTGGTCAGT CTTTGCCTAT GCTGTAGACT CATCAGGGGC CTTCCCATGC CAGTTTCCTC ATCTGTCAAA TGGCATCATT TGGGCTACTA CTGGGAGATG TAAGGAGGAA
AAAAGTCAAA TATCATGAGA TAGACTAAGG AAATAATGCT GGTGGTCTCA TGCTATGTGC CTTACTGATT TCTCTTTCAG AA IIG GAC AAA AGI IIC ICA AIG
AII AAG GAG GGI GAI IAI AAC CCC CIC IIC AII CCA GIG GCA GIC AIG GII ACI GCA IIC ICI GGG IIG GCA III AIC AII IGG CIG
GCA AGG AGA IIA AAA AAA G GTATGTGAGT TTAACTTCAC ATGAAAAGAA CACAACTTTA AAGTGAAAAA GAAAAAAAAA AGAAACCCAC AGGAAATTAA
ATGTGATAGA TTCAACACAA GCAGGATGCC AAGCTT

EXON IX (Phosphorylation Domain)

TAGTTTACA GTATTAGCAG CTGTCCCTCA AGGAAGAATC TGCAGGTAGA TGAGATGCAG ATTGGGTGGG ATAAACACTT GAATGACATA TTGGGTCTTG CCACCAGGCA
ATTTAGCAAT TCTGTCTTCT TGAGTAGCAC GGAGATGGAA TGGAACCTCA GGAGGCATCT GCATCAACAT GTCTGTTCTG TATTAGTGTC TACCACTGTT TATTAAGCCA
GTTCCTCAAA TCTCCTTTGA CACAGATAGG GTCCACCTAA CAAATACCTA ATATACTTCA AAAGACAGTT TTGAGAGTGG GAGTCTTCCT TCTCCCTTAC TTGAAAAACT
TTAAATTGTC TAATTTTTGC TAATGCCTTT TTCTCTATTT TCTATTTCAG GC AAG AAA TCC AAG AGA AG GTAAGTTTTA TTAGTGGCGA GGAGTTTCCA
CATCTGCTGA TTCATTCTCT ACTTCTTAAG TTACTTCTGC TCTAGCTAGA CACATACCCA TAGTAGTTAT TACTGGGTCT ATCAATGACA GATAGG

EXON X (Cytoplasmic Tail and 3' Untranslated Region)

ATAAGCATC ACTAAAGAGC TTGTTAGGGG TGCAGAATCT CAGGCTCCAC TCAGACCTAC TGAATCAGAG TCTGCATTTT AACACCATCT CTGAGTGGTA AGgACATGAA
AATCTGAGAA GTGCTGCTAC TAGGGTTTGC TTACATTTGT TCATCTTCAG AGGTTCCTAA AGCCTGGCCT CTTGTCTGAG ATTTCCAGCT GAAAGCATTT CCTTGCTCCT
CTTCTCATCT CTAATGAATA TTTACCTTTA CTACTAACAC TCCAAGTTTT GCAATTTTTA AACTCTTATT ATCTTTTGTT TTTCTTTCAG T ATG AAT GAC CCA
IAI IAA AICGCCCCTIG GIGAAAGAAA AIICTIGGAA IACIAAAAAI CAIGAGAICC IIIAAAICCI ICCAIGAAAC GIIIIGIGIG GIGGCAC(903 BASES)G
CAAIIIIGCA IIIGAAIAAA GCCIGCIIII IAAGIGIIAA CTAGTTTGCC TAGTTTGTTA TTTTGAAAAT TGATCATATG TTTTGTTTTC TCCCCAGTGA GTTACATGCT
CCTTCAGGGC AGAGTTTGTG TCAGATCCCT GGAGTATCTA GTGCATTACT TGACACTCAA TAAATGAATG TTCAAATAAA TCAGAAAGAG CATACAGTGC ACTGCTGATA
TAAGTTTCAG CATCCCTCTT TCTCTATGGC ATCTGATGAC CTGGGTCAGA TATCACCTAA TGTCAACAGC TGAATTC

FIG. 5C

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08467

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 31/715, 37/00, 39/395, 49/00; C12Q 1/02; G01N 33/567
US CL :424/1.1, 9, 85.8, 85.91; 435/7.24, 7.8, 29; 436/503; 530/402
According to International Patent Classification (IPC) or to both national classificati n and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.1, 9, 85.8, 85.91; 435/7.24, 7.8, 29; 436/503; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim N . |
|---|---|---|
| X,E<br>Y,<br>A, | Science, vol. 258, issued 06 November 1992, L.A. LASKY, "Selectins: interpreters of cell-specific carbohydrate information during inflamation", pages 964-969. See page 967 cols. 1-3 and page 968 cols. 1 and 3. | 6,8-9,11-12,17-18, 21,23-25<br>1-4,6-9,11-12,14-18, 20,23<br>26 |
| X<br>Y | Nature, vol. 349, issued 17 January 1991, T.A. SPRINGER ET AL, "Sticky sugars for selectins", pages 196-197. See page 196, cols. 1-2 and page 197 cols. 2-3. | 17-18,21,23<br>20 |
| X | Cell, vol. 63, issued 30 November 1990, B.K. BRANDLEY ET AL, "Carbohydrate ligands of the LEC cell adhesion molecules", pages 861-863. See page 861, col. 1. | 21 |
| Y | US, A, 5,002,873 (ST. JOHN ET AL) 26 March 1991. See col. 3 lines 1-36; col. 4, lines 18-33 and 49-55. | 1-4,6-9,11-12,14-18, 20,23 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion f the international search<br>22 December 1992 | Date of mailing of the international search report<br>08 JAN 1993 |
| Name and mailing address f th ISA/<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile N . NOT APPLICABLE | Auth rized officer<br>DAVID SAUNDERS, PRIMARY EXAMINER<br>Telephon N . (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

# INTERNATIONAL SEARCH REPORT

International application N .
PCT/US92/08467

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

I. Claims 1-10, drawn to inflamation detection in vivo using labelled LAM-1, classified in class 424/9.
II. Claims 11-16 and 24-25, drawn to body treatment methods with LAM-1, classified in class 514/2.
III. Claims 17-20 and 23-25, drawn to body treatments with LAM-1 antagonists, classified in class 514/54.
IV. Claims 21-22, drawn to assays to identify LAM-1 ligands, classified in class 435/7.24.
V. Claim 26, drawn to cell adhesion assays, classified in class 435/29.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any addtional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention frist mentioned in the claims; it is covered by claims Nos.:

**Remark n Protest**

☐ Th additional search fees were accompanied by the applicant's protest.
☐ N protest accompanied the payment f additional search fees.

F rm PCT/ISA/210 (continuation of first sheet(1))(July 1992)*